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Ribosomal Protein L7/L12 is Required for GTPase Translation Factors EF-G, RF3 and IF2 to Bind in their GTP State to 70S Ribosomes

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Abstract

Ribosomal protein L7/L12 is associated with translation initiation, elongation and termination by the 70S ribosome. The GTPase activity of EF-G requires the presence of L7/L12, which is critical for ribosomal translocation. Here, we have developed new methods for the complete depletion of L7/L12 from *E. coli* 70S ribosomes to analyze the effect of L7/L12 on the activities of the GTPase factors EF-G, RF3, IF2 and LepA. Upon removal of L7/L12 from ribosomes, the GTPase activities of EF-G, RF3 and IF2 decreased to basal levels while the activity of LepA decreased marginally. Upon reconstitution of ribosomes with recombinant L12, the GTPase activities of all GTPases returned to full activity. Moreover, ribosome binding assays indicated that EF-G, RF3 and IF2 require L7/L12 for stable binding in the GTP state, and LepA retained >50% binding. Lastly, an EF-GΔG′ truncation mutant possessed ribosome-dependent GTPase activity, which was insensitive to L7/L12. Our results indicate that L7/L12 is required for stable binding of ribosome-dependent GTPases that harbor direct interactions to the L7/L12 CTD, either through a G′ domain (EF-G, RF3) or a unique NTD (IF2). Further, we hypothesize this interaction is concomitant with counter-clockwise ribosomal intersubunit rotation, which is required for translocation, initiation and post-termination.

Keywords

ribosome translation; elongation factor G; protein L7/L12; GTPase

Introduction

Ribosomes are large, dynamic ribonucleoprotein complexes responsible for protein biosynthesis in all domains of life. Nearly all steps of translation are regulated by protein translation factors, which bind transiently to the ribosomal subunit interface [1]. Many

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Author Contributions

MAC, BGH, and AJW planned experiments, performed experiments, analyzed data, and assisted in writing the paper. CSB, CDS and MEW planned experiments, performed experiments, and analyzed data. JDW planned experiments and analyzed data. PCS planned experiments, analyzed data and wrote the paper.

translation factors are active, ribosome-dependent GTPases, which lower translation kinetic barriers through GTP hydrolysis and subsequent inorganic phosphate release [2, 3]. Several prokaryotic ribosome-dependent GTPases harbor conserved G domains (initiation factor IF2, elongation factors EF-Tu and EF-G, release factor RF3 and leader peptidase A or LepA) [4], which bind to conserved regions of the ribosomal subunit interface [5, 6], although the structural nature of GTPase binding and activity remain poorly understood.

Ribosome-dependent GTPases bind to the prokaryotic 70S ribosome complex through both the sarcin-ricin loop (SRL) and the GTPase-associated center (GAC) [5, 6]. Recent structural and mechanistic data support a model of GTPase activation whereby a phosphate oxygen from A2662 in the SRL is a strong determinant for GTP hydrolysis [6, 7]. By contrast, the role of the GAC is less understood with several ambiguous findings. The GAC, also known as the L7/L12 stalk, consists of the 1030–1124 region of the 23S rRNA, ribosomal proteins L10 and L11, and 2–3 dimers of L7/L12 (L7 is the same sequence as L12 with the addition of an N-terminal acetylation) (Figure 1A) [5]. Early reports indicated that L7/L12 was crucial for optimal translation rates, accuracy and termination [8–12], and the selective removal of L7/L12 resulted in a decrease in GTPase translation factor binding [13, 14]. More recent data have indicated that isolated L7/L12 can stimulate GTP hydrolysis by EF-G in solution [15], however, another study suggested the removal of L7/L12 from 70S ribosomes does not affect GTPase binding to ribosomal complexes, but is critical for GTPase activation and inorganic phosphate release following GTP hydrolysis [16]. Moreover, the presence of only one L7/L12 dimer resulted in active ribosomes, but multiple copies of the L7/L12 dimer are required for efficient initiation and elongation steps in translation [17].

Despite the wealth of structural data describing several 70S ribosome functional complexes, there is a relative dearth of structural definition for the L7/L12 stalk. The N-terminal domain (NTD) of each L7/L12 dimer is bound to the C terminal tail of L10 and is highly dynamic [5, 18, 19]; the flexible hinge between the NTD and C-terminal domains (CTD) of L7/L12 is required for factor binding and GTP hydrolysis [18]. Several cryo-electron microscopic reconstructions and X-ray crystal structures have illustrated direct interactions between the G′ domains of EF-G and RF3 [20–24], as well as the IF2 NTD [25], with the CTD of L7/L12, and other NMR and mutagenesis studies have elucidated conserved regions of interactions between the L7/L12 CTD and various translation factors (Figure 1B) [26]. The G′ domain is an insertion within the G domain that is highly conserved within the EF2 branch of translational GTPases (EF-G and RF3), with the exception of the GTPases LepA and BipA [27]. It has been shown that mutations within the G′ domain of EF-G lower ribosome-dependent GTPase activity, but this activity is unaffected by the presence or absence of L7/L12 [28].

While these structural data are significant, they do not reconcile the activities measured for ribosome-dependent GTPases in the presence or absence of L7/L12 in the 70S ribosome complex, specifically the activity of GTPases that do not harbor a G′ domain, such as LepA [29]. LepA is a ribosome-dependent GTPase that is highly conserved amongst prokaryotes, mitochondria and chloroplasts [27]. Initially described as an elongation factor that back translocates tRNA [30], recent data strongly suggest LepA principally contributes to

translation initiation [31]. The role of LepA in initiation has been further characterized in a new study that suggests LepA plays a direct role in late stage biogenesis of the 30S subunit through nascent 70S complex assembly, which “test drives” mature ribosome particles [32]. In contrast to EF-G, RF3 and IF2, there are presently no data to suggest direct interactions between LepA and ribosomal protein L7/L12. Finally, a recent study suggests that EF-G catalyzes a proline switch within L11, allowing for a direct interaction between the L11 NTD and L12 CTD, which further complicates the interpretation of reported data [33, 34].

The majority of studies addressing the role of L7/L12 in GTPase activity have depended on an established salt/ethanol extraction method for the selective removal of L7/L12 from the 70S ribosome [35–37], which generates 70S core particles that are mostly depleted of L7/L12 (the extent of L7/L12 removal varies; one study reports ~15% residual L7/L12 following the depletion protocol) [36]. In the presence of an active ribosome-dependent GTPase, L7/L12 functions catalytically, as the active GTPase rapidly dissociates following GTP hydrolysis and phosphate release [38]. Moreover, L7/L12 exchanges at a relatively fast rate in the context of ribosome stability and GTPase activity assays (~50% exchange in two hours) [39]. Provided with these observations, a method for complete removal of L7/L12 is required to clarify the role of the L7/L12 protein in GTPase function. In this report, we have developed improved procedures for the generation of 70S ribosomes completely depleted of L7/L12 and a highly pure fraction of recombinant L12 for reconstitution studies. Subsequent GTPase activity and binding studies with EF-G, IF2, RF3 and LepA have served to delineate the role of L7/L12 in ribosome-dependent GTPase activity.

Results

Analysis of improved purification for recombinant ribosomal protein L12 and L7/L12-depleted 70S ribosomes

Ribosomal protein L12 was subcloned into an expression vector harboring an N-terminal hexahistidine tag, which allowed for expression and purification of L12 by immobilized metal affinity chromatography (IMAC) (Figure 2A). Following initial purification steps, L12 was greater than 95% pure, however, the purified L12 fraction possessed contaminating GTPase activity. In a malachite green GTPase assay, L12 alone resulted in 15% activity relative to EF-G-dependent GTPase activity in the presence of 70S ribosomes as described below. To remove the GTPase contamination from L12, the protein was denatured while bound to the IMAC column by the addition of 7M urea. L12 was subsequently eluted with imidazole in the denatured state and refolded by dialysis in L12 purification buffer (Figure 2A). This highly purified fraction of L12 was soluble to at least 2 mg/mL, but lacked any measurable GTPase activity. Moreover, L12 did not appear to bind EF-G or catalyze EF-G-dependent GTPase activity in the absence of 70S ribosomes, in contrast to previous findings [15].

To assess whether the purified L12 was in a native, folded state, further analysis was performed by circular dichroism (CD) and size exclusion chromatography (SEC) (Figure 2B,C). The CD spectrum of refolded L12 resulted in negative ellipticity at 220 nm, which was indistinguishable from L12 purified in its native state, indicating that L12 was in a

soluble, folded state. Furthermore, L12 eluted from a Superdex 75 SEC column with an elution volume consistent with L12 existing as a dimer in solution.

The selective removal of ribosomal protein L7/L12 from 70S ribosomes was performed with an ammonium chloride incubation step and subsequent ethanol precipitation, as previously described [35–37]. Following this initial removal step, residual L7/L12 remained present as measured by silver stained SDS-PAGE gel as well as Western blot (Figure 2D,E). This preparation of L7/L12-depleted 70S ribosomes harbored EF-G-dependent GTPase activity between 5 and 30% depending on the preparation, which is consistent with previous studies [28, 36].

To remove the residual L7/L12-containing 70S ribosomes, we performed the initial 70S purification steps with the JE28 *E. coli* cell line that was previously developed for improved 70S purification [40]. The JE28 cell line harbors a chromosomally encoded hexahistidine tag on the C-terminus of ribosomal protein L12, allowing for IMAC purification of 70S ribosomes. Following 70S affinity purification and subsequent removal of L7/L12, as described above, the depleted 70S fraction was further purified with a second IMAC column (Figure 2F). The ribosome fraction that is completely devoid of L7/L12 elutes in the flow-through of the second IMAC column while any remaining L7/L12-containing 70S remain bound until the addition of an imidazole-containing elution buffer. Western blot analysis indicates that the hexahistidine-tagged L7/L12 was completely removed (Figure 2E). Moreover, there is no detectable GTPase activity for the completely L7/L12-depleted 70S ribosomal preparation (Figure 3A).

Elongation Factor G requires ribosomal protein L12 for ribosome-dependent GTPase activity and stable binding to the 70S ribosome in the GTP state

The effect of ribosomal protein L12 on the ribosome-dependent GTPase activity of EF-G was evaluated with purified 70S ribosomes, L7/L12-depleted 70S particles, and recombinant L12 and EF-G. GTPase reactions were performed *in vitro* by employing a malachite green colorimetric assay as previously described for the measurement of ribosome-dependent GTPase activities (Figure 3A) [41]. In the presence of 70S ribosomes, the GTPase activity of EF-G increased dramatically as expected. Once L7/L12 was completely removed from 70S ribosomes as described above, the GTPase activity of EF-G was indistinguishable from EF-G alone, which is negligible by comparison. Upon reconstitution of 70S ribosomes with recombinant L12, the ribosome-dependent GTPase activity of EF-G returned to levels similar to complete 70S ribosomes. These data also suggest that the purification of L7/L12-depleted ribosomes and refolded recombinant L12 did not significantly perturb the structure or activity of either component.

To investigate the mode of activation by L12, the effect of its absence on the stable binding of EF-G in the presence of a non-hydrolyzable analog of GTP (GDPNP) to 70S ribosome complexes was determined by three different *in vitro* assays. First, we employed a binding assay that relies on gel filtration (Figure 3B). Briefly, EF-G and GDPNP were incubated with 70S ribosomes in the presence or absence of ribosomal protein L7/L12. The EF-G/ribosome complexes were subsequently applied to a gel filtration resin, eluted by centrifugation and analyzed by SDS-PAGE. Ribosomal complexes eluted from the resin

while unbound EF-G was retained. In the presence of endogenous 70S ribosomal complexes, EF-G co-eluted when GDPNP was present, indicative of stable binding, as previously described [42]. Once L7/L12 was completely removed, however, EF-G in the presence of GDPNP eluted only a minor amount from the resin (20%), suggesting that EF-G may bind with a low affinity, as previously described [43–45]. When recombinant L12 was added to reconstitute complete 70S ribosomes, EF-G•GDPNP bound as before, consistent with other observations described herein. To substantiate these results, a second ribosome-binding assay was employed. In this assay, 70S ribosome/EF-G complexes were formed as described above and then added to a sucrose cushion and subsequently ultracentrifuged (Figure 3C). While this method also measures equilibrium binding, the 70S/GTPase complexes diffuse during centrifugation, thus allowing low affinity complexes to dissociate. Ribosomal complexes with stably bound EF-G pelleted to the bottom of the ultracentrifuge tube while unbound EF-G did not migrate through the sucrose solution. The resulting pellets were resuspended and analyzed by SDS-PAGE. In the presence of endogenous L7/L12 with the 70S ribosome, EF-G•GDPNP pelleted with ribosomes, suggestive of stable binding. Upon removal of L7/L12, EF-G did not co-migrate, but EF-G binding was restored following the addition of recombinant L12, which is consistent with the gel filtration data described above.

In the third assay, a quantitative method for the binding of EF-G•GDPNP to 70S ribosomes in the presence and absence of L7/L12 was performed. In this assay, a fluorescein label was site-specifically labeled on domain IV of EF-G, and the quantum yield of the fluorescein is diminished upon 70S ribosome binding, as shown previously [46]. In the presence of complete 70S ribosomes, the equilibrium dissociation constant for EF-G•GDPNP was determined to be 23 nM, whereas the binding of EF-G•GDPNP could not be measured for L7/L12-depleted 70S ribosomes, as expected (Figure 3D). Taken together, these data indicate that EF-G requires the presence of ribosomal protein L7/L12 for stable binding and GTPase activity.

Ribosomal protein L12 has differential effects for various ribosome-dependent GTPases

To further characterize the role of ribosomal protein L12 on the ribosome-dependent activation of GTPase activity for translation factors, GTPase activity and ribosome binding were assessed for three other ribosome-dependent GTPases, namely initiation factor 2 (IF2), release factor 3 (RF3) and leader peptidase A (LepA) (Figure 4A,D,G). Each of these GTPase translation factors harbor conserved G domains and possess ribosome-dependent GTPase activity as described in other studies [42, 47–49]. In experiments with both IF2 and RF3, GTPase activity was dramatically increased upon the addition of 70S ribosomes, albeit at a slower rate than what was observed for EF-G. In the presence of L7/L12-depleted 70S ribosomes, both IF2 and RF3 maintained GTPase activities that were indistinguishable from the basal rates in the absence of ribosomes, similar to the observed behavior for EF-G. Upon reconstitution of 70S ribosomes with recombinant L12, however, the ribosome-dependent GTPase activity for IF2 and RF3 returned to near 100% relative to endogenous 70S ribosomes. Remarkably, LepA displayed significantly different behavior with regards to ribosome-dependent GTPase activity. In the presence of endogenous 70S ribosomes, the GTPase activity of LepA was rapid and approached 100% on a similar timescale as EF-G. Following the removal of L7/L12, the ribosome-dependent GTPase activity for LepA

dropped marginally, retaining greater than 50%, distinct from all other GTPases assayed in this study. Once recombinant L12 was added to reconstitute the entire 70S ribosome complex, the GTPase activity of LepA returned to near 100%, as expected based on the other GTPases in this study. One possible reason for the ribosome-dependent GTPase activity of LepA with L7/L12-depleted 70S ribosomes would be contamination of the LepA purification with endogenous L12. To address this, we did not observe any protein SDS-PAGE contaminants in the 10–15 kDa range of the LepA purifications. The observed GTPase activity of LepA with L7/L12-depleted ribosomes was indistinguishable with LepA purifications that included an additional gel filtration step. Lastly, we observed no direct binding between recombinant L12 and LepA by Ni-NTA affinity pulldown and fluorescence quenching assays.

Following the GTPase activity results for IF2, RF3 and LepA, gel filtration and ultracentrifugation assays were performed to measure ribosome binding in the presence and absence of ribosomal protein L12 (Figure 4). In the presence of endogenous 70S ribosomes, both IF2 and RF3 co-eluted with the 70S fraction similar to the observed binding for EF-G. For the L7/L12-depleted 70S complexes, IF2 and RF3 failed to co-elute with the 70S fraction, thereby remaining in the gel filtration resin and indicating a lack of stable binding for either IF2 or RF3 with the 70S ribosome in the absence of L7/L12 (Figure 4B,E). When ribosome binding was measured for both IF2 and RF3 by ultracentrifugation, similar results were observed. Briefly, IF2 and RF3 bound to complete 70S ribosomal complexes but failed to bind upon removal of endogenous L7/L12. Upon reconstitution with recombinant L12, near 100% binding to 70S ribosomes returned, consistent with the observed binding behavior for EF-G (Figure 4C,F). These results suggest that L12 is essential for ribosome binding in the GTP state and ribosome-dependent GTPase activation for the conserved translation factor GTPases, EF-G, IF2 and RF3.

Consistent with the observed ribosome-dependent GTPase activity for LepA, the observed ribosome binding behavior was strikingly different from the other translation factors assayed in this study. In the presence of endogenous 70S ribosomes, LepA bound stably as measured by both gel filtration and ultracentrifugation, whereby the majority of 70S ribosomes were bound as quantified by bands on the resultant SDS-PAGE gels. After the endogenous L7/L12 was removed by ammonium chloride incubation, ethanol precipitation and IMAC separation as was performed for all other GTPase binding experiments, LepA retained greater than 50% binding to 70S ribosomes as measured by both assays (Figure 4H,I). Lastly, upon incubation with recombinant L12 to reconstitute the complete 70S ribosomal complex, LepA binding was restored to levels similar to endogenous 70S ribosomes (Figure 4I). While the trends of LepA are reminiscent of the binding and activation behavior of the other GTPases in this study, the quantitative measure of LepA was distinct from EF-G, IF2 and RF3. Structural reasoning for these observations likely must concern interactions beyond the conserved G domains for each GTPase translation factor, which will be described below.

Removal of the G′ domain from EF-G abrogates the stimulatory effect of L7/L12 in ribosome-dependent GTPase activity

To substantiate the observations above that ribosomal protein L7/L12 is required for the GTP hydrolysis and stable binding in the GTP state for ribosome-dependent GTPases harboring a G′ domain, a G′ domain truncation mutant of EF-G was cloned and purified (EF-GΔG′). The purified EF-GΔG′ protein was found to be soluble to at least 2 mg/mL and folded based on CD analysis (Figure 5B). Similar to previous observations, the EF-GΔG′ mutant possessed ribosome-dependent GTPase activity, albeit at an approximately 5-fold lower level than wild type EF-G in the presence of complete 70S ribosomes (Figure 5A) [28, 48]. Upon the addition of EF-GΔG′ to L7/L12-depleted 70S ribosomes, the GTPase activity was similar to that of EF-GΔG′ in the presence of complete 70S ribosomes, indicating the ribosome-dependent GTPase activity of EF-GΔG′ is insensitive to the presence or absence of ribosomal protein L7/L12.

To further address the nature of interactions between ribosomal protein L7/L12 and GTPases harboring a G′ domain, titrations of EF-G and LepA were performed relative to a constant concentration of 70S ribosomes in the presence or absence of L7/L12. In titrating from 0.05 to 5 μM GTPase relative to a constant concentration of 0.2 μM 70S ribosomes, EF-G and LepA approach maximal activity at 2.5 μM in the presence of L7/L12. For L7/L12-depleted 70S ribosomes, LepA approaches 80% activity at 5 μM while EF-G only reaches 20%. These data further support the finding that LepA displays high levels of ribosome-dependent GTPase activity in the absence of L7/L12 while EF-G does not.

Discussion

The binding and activation of ribosome-dependent GTPases is a central regulatory process in translation. While the binding of each GTPase is mutually exclusive, each translation factor appears to bind uniquely to the ribosomal subunit interface to perform their distinct functions. For each GTPase that possesses either a G′ domain (EF-G and RF3) or an N-terminal extension (IF2), structural evidence illustrates a direct connection between these translation factors and the CTD of ribosomal protein L7/L12 [20–25]. Previous studies had initially demonstrated the importance of ribosomal protein L7/L12 for EF-G function [14], whereby selective removal of L7/L12 resulted in >1000-fold decrease in GTPase activity and severely decreased binding [8–10, 13]. Subsequent studies based on L7/L12 mutants assert that the presence L7/L12 is primarily responsible for inorganic phosphate release by EF-G following GTP hydrolysis [36]. In this study, we developed an improved protocol for the purification of L7/L12-depleted 70S ribosomes to reconcile these studies and further understand the role of L7/L12 in GTPase binding and activation (Figure 2). Our results indicate that L7/L12-depleted 70S ribosomes are unable to catalyze EF-G-dependent GTP hydrolysis, and EF-G does not stably bind to L7/L12-depleted ribosomes in its GTP state (Figure 3).

To extend our understanding of this interaction, we examined the role of L7/L12 in GTPase activity and binding with other ribosome-dependent GTPases. The results herein illustrate that the translation factors RF3 and IF2 show similar effects to that of EF-G, whereby L7/L12 is required for GTPase stable binding in the GTP state and subsequent GTP

hydrolysis. By contrast, LepA does not have such a dependence on the presence of L7/L12, resulting in greater than 50% binding and GTPase activity (Figure 4).

The majority of available data relating the presence of L7/L12 to the activity of ribosome-dependent GTPases involves EF-G [5, 15, 16, 18, 28, 36, 39]. A series of point mutations to highly conserved residues hypothesized to interact directly with EF-G affect both the K_M and k_{cat} for EF-G-catalyzed GTP hydrolysis, which indicates that L7/L12 contributes to both EF-G binding and GTPase transition state stabilization [36]. By comparison, point mutations in the G′ domain of EF-G that were predicted to form direct contacts with the L7/L12 CTD displayed large defects of GTP hydrolysis activity [28]. Moreover, an NMR study suggested direct interactions between EF-G, EF-Tu, RF3 and IF2 and several conserved residues on the L12 CTD [26]. In this study, we generated a G′ domain truncation mutant (EF-GΔG″) to measure how the GTPase activity of this mutant was dependent on the presence of L7/L12. The overall GTPase activity of the EF-GΔG′ mutant in the presence of 70S ribosomes was significantly less than that of wild type EF-G, but this diminished activity was unaffected by the selective removal of ribosomal protein L7/L12 (Figure 5).

A key structural finding that is consistent between EF-G, RF3 and IF2 is that each GTPase binds 70S ribosomes in a counterclockwise rotation [21–25, 50–53], which is concomitant with direct connections to the L12 CTD. In contrast to these structural observations, LepA-bound ribosomes have been reported to be in either an unrotated or clockwise-rotated 70S conformation, and no connection between LepA and L7/L12 has been observed [47, 54]. By relating these structural differences to the data presented in this study, we hypothesize that the L7/L12 CTD contributes a key role in conformational changes within each GTPase that harbors a direct contact to it, thus also rotating the 70S ribosome counterclockwise. It is our hypothesis that the EF-GΔG′ mutant lacks the ability to stabilize the counterclockwise rotated state, primarily due to its lack of direct interaction with the L12 CTD, thus resulting in its GTPase activity being independent of the presence of L7/L12. In contrast, the clockwise rotation stabilized by LepA in its GTP state may play a unique role in late stage ribosome biogenesis. Gibbs and colleagues propose that LepA binds to precursor 70S particles as a quality control mechanism that precludes active translation by mature 70S ribosomes [32].

In this study, we demonstrated a novel method for improved purification of L7/L12-depleted ribosomes and recombinant ribosomal protein L12. These highly purified ribosome components were subsequently employed to determine the role of L7/L12 in ribosome-dependent GTPase binding and activity. Taken together, the results reported in this study are consistent with previous studies of EF-G and clarify that the presence of L7/L12 is a critical component to allow stable binding of EF-G in the GTP state. These studies have now also been extended to other ribosome-dependent GTPases and have illustrated the importance of direct connections of GTPases, through either the G′ domains of EF-G and RF3 or the NTD of IF2, to the L7/L12 CTD to adopt a ‘GTPase active’ conformation, which has implications to 70S ribosomal conformations during different steps of translation.

Materials and Methods

Reagents

Guanosine-5′-triphosphate (GTP), guanosine-5′-diphosphate (GDP), guanosine-5′-(β,γ-imino) triphosphate (GDPNP) and Sephacryl S-300 HR resin were purchased from Sigma. HisPur Ni-NTA Superflow Agarose resin was purchased from Thermo Scientific. TALON resin was purchased from Clontech. Malachite Green dye was purchased from Fisher Scientific.

Preparation of ribosomes and GTPases

Ribosomes were grown and purified from *E. coli* JE28 cells as previously described [40, 42]. Briefly, JE28 cells were grown at 37°C until mid-log phase was reached, then placed in an ice bath until the temperature reached 4°C. Cells were pelleted by centrifugation and resuspended in JE28 lysis buffer (20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl, 5 mM imidazole, 1 mM dithiothreitol). Once resuspended, cells were lysed by sonication and centrifuged at 18,500 rpm and 4°C for 1 hour in a SS34 rotor. The soluble fraction was filtered through a 5 μm syringe filter, followed by a 0.2 μM sterile syringe filter and subsequently purified with TALON metal affinity resin (JE28 wash buffer: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 500 mM NH_4Cl, 5 mM imidazole, 1 mM dithiothreitol; JE28 elution buffer: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl, 150 mM imidazole, 1 mM dithiothreitol). The eluent was dialyzed in JE28 salt wash buffer (20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl, 1 mM dithiothreitol) overnight and subsequently pelleted twice at 150,000xg (60Ti rotor, 57,400 rpm, 2 hours, 4°C). The final ribosome pellet was resuspended in a small volume of ribosome storage buffer (50 mM Tris–HCl (pH 7.5), 30 mM NH_4Cl, 7 mM $MgCl_2$, 25% (v/v) glycerol), flash frozen in liquid nitrogen and stored at −80°C.

All GTPases (EF-G, LepA, IF2, and RF3) were initially cloned into a pSV281 vector as previously described [42] and expressed in *E. coli* BL21(DE3) cells through the addition of 0.3–0.6 mM IPTG, and grown at 15°C for 12–18 hours while shaking. Cells were pelleted at 7500 rpm in a GS3 rotor at 4°C for 10 minutes, and resuspended in GTPase lysis buffer (50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$, 15 mM imidazole, 25% (v/v) glycerol, 7 mM 2-mercaptoethanol). Cells were lysed by sonication, then clarified by centrifuging twice at 18,000 rpm, 4°C, for 45 minutes in an SS34 rotor. If further clarification was necessary, the supernatant was then filtered through a 5 μm, then a 0.45 μm sterile syringe filter. GTPases were incubated with Ni-NTA resin for at least 30 minutes prior to washing and eluting (GTPase wash buffer: 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 60 mM NH_4Cl, 7 mM $MgCl_2$, 15 mM imidazole, 25% (v/v) glycerol, 7 mM 2-mercaptoethanol; GTPase elution buffer: 50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$, 250 mM imidazole, 25% (v/v) glycerol, 7 mM 2-mercaptoethanol). Purified GTPases were first dialyzed overnight in GTPase storage buffer (50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$, 25% (v/v) glycerol, 7 mM 2-mercaptoethanol), then concentrated using a 15 kDa MWCO spin concentrator, until concentrations reached acceptable levels. Purified, concentrated GTPases were flash frozen in liquid nitrogen and stored at −80°C.

Expression, purification and analysis of ribosomal protein L12

The *E. coli* L12 gene was cloned into the pSV281 vector using *Bam*HI (5′) and *Xho*I (3′) restriction sites, introducing an N-terminal $(His)_6$-tag. The L12 protein was expressed by the addition of 0.5 mM IPTG at mid-log phase and grown for an additional three hours at 37°C. Affinity purification of L12 was performed identically to the GTPases described above, with the addition of 7M urea to each buffer after the protein was immobilized on the resin. Following elution, the purified L12 fraction was centrifuged at 150,000xg for 2 hours at 4°C. The supernatant was refolded through slow dialysis in two separate 1 L vessels of GTPase storage buffer for 24 hours each. The purified, refolded protein was concentrated using a 10 kDa MWCO spin concentrator and purity was assessed by SDS-PAGE. The concentration of L12 was determined with a Bradford assay. Purified, concentrated aliquots were flash frozen and stored at −80°C. Circular dichroism (CD) spectra were collected on an Olis DSM 10 spectropolarimeter. Prior to data collection, L12 was diluted to 0.5 mg/mL in reaction buffer (50 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $MgCl_2$). Ellipticity was monitored from 200 to 270 nm in 1 nm increments at 20°C. Lastly, the refolded, purified fraction of L12 was analyzed by size exclusion chromatography with a Superdex 75 column in reaction buffer. Protein L12 was also purified in its native state following the procedures listed above for GTPase purification.

L7/L12 depletion and reconstitution of 70S ribosomes

Ribosomal protein L7/L12 was selectively depleted from *E. coli* 70S ribosomes with procedures previously described [35, 36]. All solutions were stored at 4°C for 24 hours prior to depletion, and 70S ribosomes were thawed on ice immediately prior to depletion. In a microfuge tube, 450 pmol of purified $(His)_6$-L12 tagged 70S ribosome were diluted to 450 μL in L12 extraction buffer (20 mM Tris-HCl (pH 7.5), 1 M NH_4Cl, 20 mM $MgCl_2$, 50% (v/v) glycerol, 5 mM 2-mercaptoethanol) and allowed to incubate for 5 minutes on ice. To the L12 extraction mixture was added 250 μL of ice-cold 100% ethanol, and the solution was stirred at 4°C for 5 minutes, at which point another 250 μL aliquot of 100% ethanol was added. After an additional 5 minutes of stirring, the mixture was centrifuged at 150,000xg for 45 minutes at 4°C. Initial L7/L12-depleted ribosome pellets were resuspended in 100 μL ribosome storage buffer and applied to a 5 mL Ni-NTA column. Completely L7/L12-depleted ribosomes were collected in the initial flowthrough in ribosome storage buffer, while non-depleted ribosomes were eluted using JE28 elution buffer and discarded. The reintroduction of purified L12 to L7/L12-depleted 70S ribosomes was accomplished through incubation of depleted 70S ribosomes with a 5-fold molar excess of purified $(His)_6$-tagged L12 at 37°C for 30 minutes in ribosome storage buffer.

GTPase activity assay

Malachite green (0.045% (w/v) malachite green dye in ddH_2O) was combined with 4.2% (w/v) ammonium molybdate in a 3:1 ratio, and stirred for 30 minutes at room temperature and subsequently sterile filtered (0.45 μm) to generate the reaction dye. GTPase activity assays were performed by the addition of GTPase (5 μM), 70S (0.2 μM), L7/L12-depleted 70S ribosomes (70SΔL12 (0.2 μM), 70SΔL12+L12 (0.2 μM) in GTPase reaction buffer (90 mM K-HEPES (pH 7.5), 100 mM NH_4Cl, 20 mM $Mg(CH_3COO)_2$, 1 mM DTT). Each

reaction was incubated at room temperature for 15 minutes before GTP (25 μM) was added to each reaction, which was further incubated at room temperature for an additional 15 minutes. Reactions were quenched through the addition of malachite green reagent, and color was allowed to develop for an additional 10 minutes. Colorimetric quantification of released phosphate was performed in 96 well plates at 620 nm using a BioTek Epoch plate reader.

GTPase binding assays

Stable binding of GTPases to 70S ribosomes was assayed with two different qualitative methods: a size exclusion/centrifugation protocol [42], and a sucrose cushion ultracentrifugation protocol [41]. Initially, ribosome functional complexes were formulated in GTPase reaction buffer with 1 μM 70S, 5 μM GTPase, and 0.5 mM GDPNP and incubated at 37°C for 20 minutes. For the size exclusion assay, each 60 μL reaction was added to 750 μL Sephacryl-300 HR resin that had been pre-equilibrated in GTPase binding buffer (80 mM HEPES (pH 7.5), 100 mM NH_4Cl, 20 mM $Mg(CH_3COO)_2$) with 0.5 mM GDPNP in a spin column. The column was immediately centrifuged at 1700xg for 2 minutes. The flow-through was collected, precipitated with cold acetone, and subsequently analyzed via SDS-PAGE. For the sucrose buffer ultracentrifugation assay, reactions were layered on top of a 10% (w/v) sucrose solution (in GTPase reaction buffer with 0.5 mM GDPNP) and centrifuged at 255,000xg for 10 minutes at 4°C. Pellets were resuspended in 20 μL ddH_20 by gentle vortex and analyzed by SDS-PAGE. To quantitate the extent of GTPase binding to 70S ribosomes, the intensity of each GTPase band in the SDS-PAGE gel was measured and normalized to the intensity of ribosomal protein L1, which serves as a loading control. A third quantitative fluorescence-based assay was employed to measure equilibrium binding of EF-G to 70S ribosomal complexes [46]. A mutant EF-G containing a single cysteine at position 591 in domain IV (EF-G-591) was initially coupled with 5-iodoacetomido fluorescein (5-IAF), as previously described. EF-G-591 was buffer exchanged to the coupling buffer (10 mM HEPES-KOH (pH 7.8), 100 mM KCl) and the coupling reaction was carried out with a 1:5 molar ratio of EF-G-591 to 5-IAF in the presence of 14 μM 2-mercaptoethanol overnight at room temperature, which was quenched the following day with 54 mM 2-mercaptoethanol. Ribosome functional complexes were initially formulated in fluorescent binding buffer (50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 1.6 mM DTT, 1 mM GDPNP) with 1.5 molar excess $tRNA^{Phe}$ and mRNA 32 (5′-GGCAAGGAGGUA AAAAUGUUUAAAGGU AAAUCUACU-3′) and incubated for 30 minutes at 37°C. With an EF-G-591/5-IAF concentration of 20 nM, ribosomal complexes were titrated from 600 nM to 0 nM in 50 μL reactions. Fluorescence measurements were recorded with a Perkin Elmer $Victor^3V$ fluorescence plate reader and K_D measurements were calculated with a 1:1 binding fit using GraphPad Prism.

Site-directed mutagenesis, expression and purification of EF-GΔG′

The G′ domain of EF-G was truncated from the pSV281 expression plasmid encoding *E. coli* EF-G with QuikChange Lightning site-directed mutagenesis according to the manufacturer's instructions (Agilent, Santa Clara, CA). Based on molecular modeling with the *T. thermophilus* EF-G (pdb#: 2EFG), the alpha carbons of E. coli residues 166 and 261 were within 5 Å of each other and solvent-exposed upon the removal of the G≥ domain, thus

residues 167–260 of the EF-G sequence were deleted with the following primers: (*forward*) 5′-CGCTGCAGCTGGCGTGTGGTTCTGCGTT-3′, (*reverse*) 5′-AACGCAGAACCACACGCCAGCTG CAGCG-3′. The resultant EF-GΔG′ expression plasmid was subsequently transformed into NiCo21 *E. coli* cells for protein overexpression. The transformed cells were grown to mid-log phase at 37°C followed by cooling to 20°C, induction with 400 μM IPTG and incubation for 8 hours before harvesting by centrifugation. The EF-GΔGλ protein was purified similarly to full-length EF-G as described above with the only difference being the use of TALON resin for the initial IMAC purification followed by further purification with a monoQ column. The purified EF-GΔG′ protein was concentrated to 2 mg/mL and analyzed by CD as described above.

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Abbreviations

EF-G	elongation factor G
RF3	release factor 3
IF2	initiation factor 2
LepA	leader peptidase A
L7/L12	ribosomal protein L7/L12
GTPase	guanosine 5′ triphosphate hydrolase
GDPNP	guanosine 5′-[β,γ-imido] triphosphate

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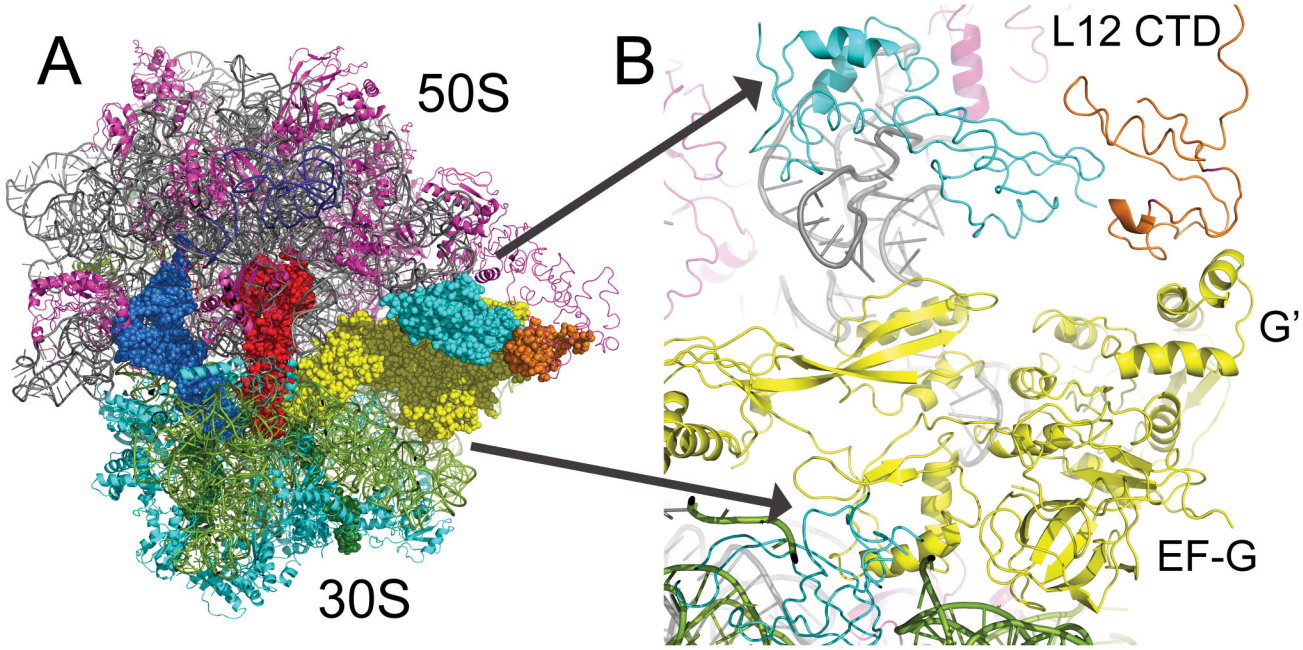

Figure 1. X-ray crystal structure of the 70S/EF-G complex

(A) ribbon diagram representation of the top view for the 70S ribosomal subunit interface with EF-G bound (PDBID#: 4W29, gray: 23S rRNA, green: 16S rRNA, magenta: 50S ribosomal proteins, cyan: 30S ribosomal proteins, red: P-site tRNA, blue: E-site tRNA, yellow: EF-G, cyan: ribosomal protein L11, orange: ribosomal protein L7/L12 C-terminal domain). **(B)** Ribbon diagram representation of the direct interactions between EF-G and the GTPase-associate center. The G′ domain of EF-G forms a direct contact with the CTD of L7/L12.

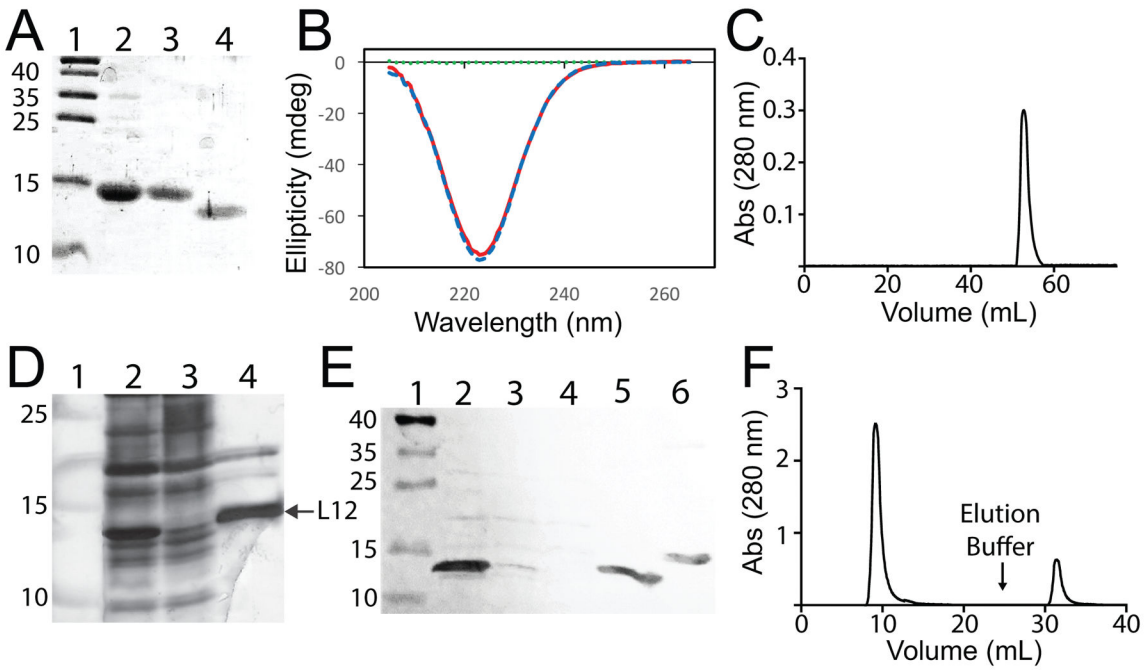

Figure 2. Purification of recombinant protein L12 and L7/L12-depleted 70S ribosomes

Removal of C-terminal tagged L12 from 70S ribosomes and purification of N-terminal tagged L12. **(A)** SDS-PAGE of recombinant L12 before and after unfolding with urea. Lanes: (1) MW standards, (2) initial purification of recombinant protein L12, (3) recombinant protein L12 purified in the unfolded fraction, (4) protein L7/L12 depleted from 70S ribosomes (migration differs due to different hexahistidine tags). **(B)** Far UV CD spectra of purified, recombinant protein L12. Green line (dotted): buffer, red line (solid): L12 purified under native conditions, blue line (dashed): denatured and refolded L12. **(C)** Superdex 75 chromatograph of refolded protein L12 in native folding buffer. **(D)** Silver-stained SDS-PAGE of L12 depletion supernatant. Lanes: (1) MW standards, (2) 70S ribosomes, (3) L7/L12-depleted 70S ribosomes, (4) acetone precipitation of removed L7/L12. **(E)** Western blot of L12 or L7/L12 fractions with an anti-hexahistine tag antibody. Lanes: (1) MW standards, (2) 70S ribosomes, (3) initial L7/L12-depleted 70S ribosomes, (4) complete L7/L12-depleted 70S ribosomes, (5) removed ribosomal proteins following depletion protocol, (6) recombinant protein L12. **(F)** FPLC chromatograph of the Ni-NTA secondary purification procedure for L7/L12-depleted 70S ribosomes (arrow: buffer change to elution buffer).

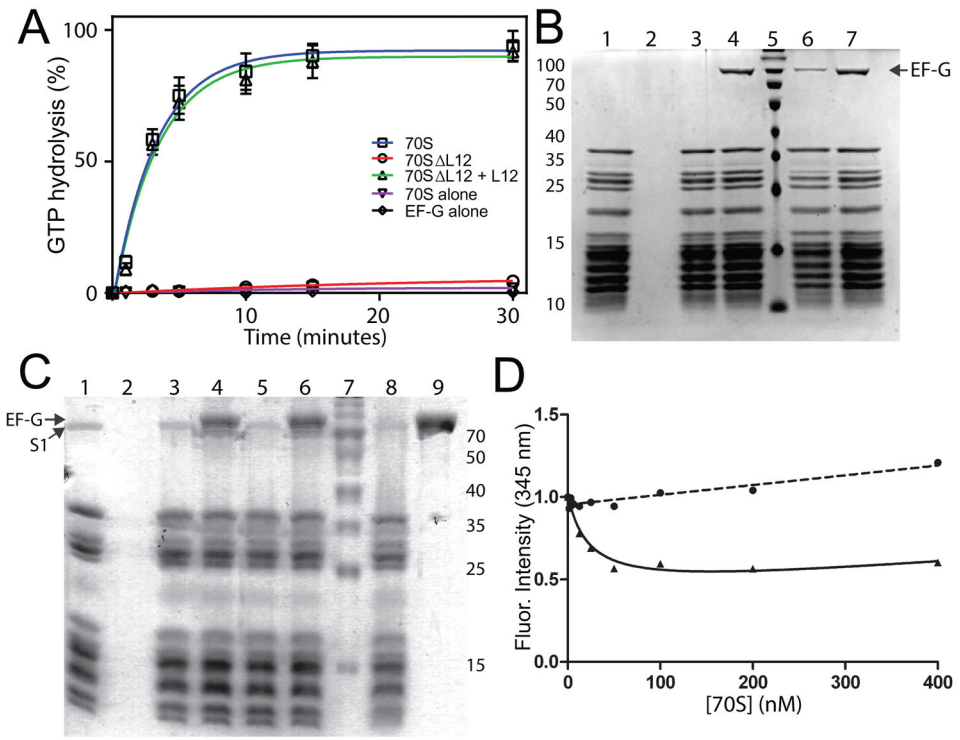

Figure 3. The effect of L7/L12 removal from 70S ribosomes on the activity of EF-G

(A) Malachite green GTP hydrolysis activity assay. Squares (blue): 70S/EF-G/GTP, circles (red): 70SΔL12/EF-G/GTP, triangles (green): 70SΔL12/recombinant L12/EF-G/GTP, inverted triangles (purple): 70S/GTP, diamonds (black): EF-G/GTP. Each data point measured in triplicate and error bars represent standard deviations from the mean. **(B)** Gel filtration-based ribosome binding assay for EF-G. Lanes: (1) 70S ribosomes, (2) EF-G, (3) 70S/EF-G, (4) 70S/EF-G/GDPNP (5) MW standards, (6) 70SΔL12/EF-G/GDPNP, (7) 70SΔL12/recombinant L12/EF-G/GDPNP. **(C)** Ultracentrifugation-based ribosome binding assay for EF-G. Lanes: (1) 70S ribosomes, (2) EF-G, (3) 70S/EF-G, (4) 70S/EF-G/GDPNP, (5) 70SΔL12/EF-G/GDPNP, (6) 70SΔL12/recombinant L12/EF-G/GDPNP, (7) MW standards, (8) 70S control, (9) EF-G control. **(D)** Fluorescence quenching-based EF-G binding assay. Solid line: 70S/EF-G/GDPNP (K_D = 23±11.3 nM), dashed line: 70SΔL12/EF-G/GDPNP (K_D = not determined).

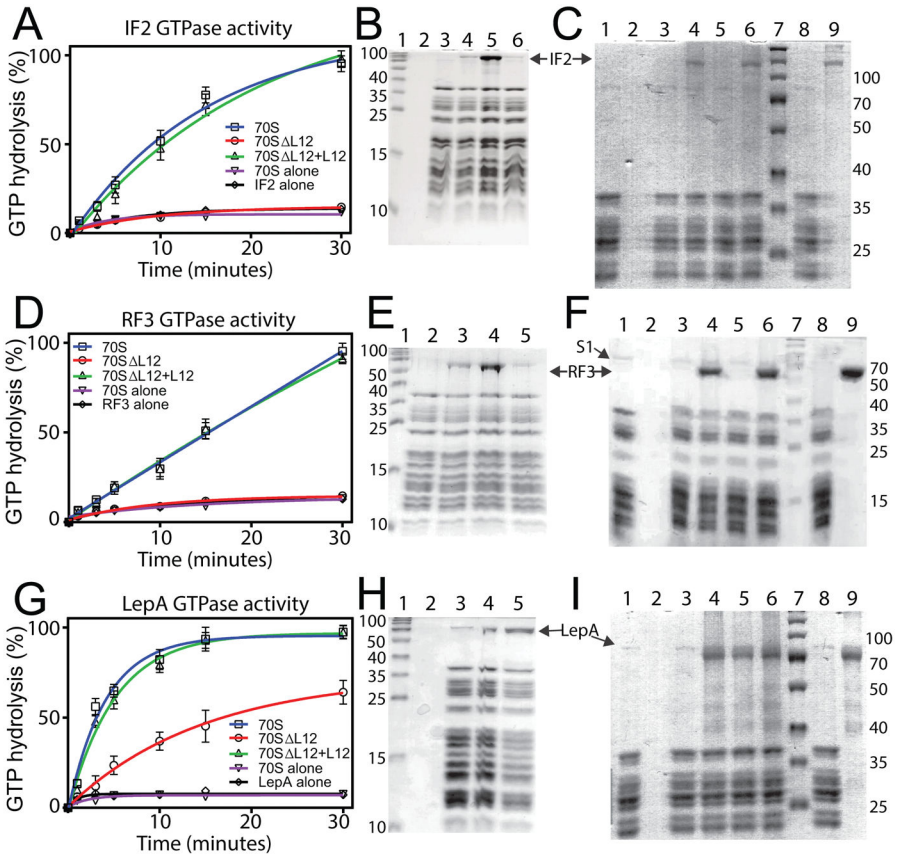

Figure 4. The effect of L7/L12 removal from 70S ribosomes on the activities of RF3, IF2 and LepA

(A, D, G) Malachite green GTP hydrolysis activity assay for IF2 (A), RF3 (D) and LepA (G). Squares (blue): 70S/GTPase/GTP, circles (red): 70SΔL12/GTPase/GTP, triangles (green): 70SΔL12/recombinant L12/GTPase/GTP, inverted triangles (purple): 70S/GTP, diamonds (black): GTPase/GTP. Each data point measured in triplicate and error bars represent standard deviations from the mean. **(B)** Gel filtration-based ribosome binding assay for IF2. Lanes: (1) MW standards, (2) IF2, (3) 70S ribosomes, (4) 70S/IF2/GTP, (5) 70S/IF2/GDPNP, (6) 70SΔL12/IF2/GDPNP. **(E)** Gel filtration-based ribosome binding assay for RF3. Lanes: (1) MW standards, (2) 70S ribosomes, (3) 70S/RF3/GTP, (4) 70S/RF3/GDPNP, (5) 70SΔL12/RF3/GDPNP. **(H)** Gel filtration-based ribosome binding assay for LepA. Lanes: (1) MW standards, (2) LepA, (3) 70S/LepA/GTP, (4) 70S/LepA/GDPNP, (5) 70SΔL12/LepA/GDPNP. **(C, F, I)** Ultracentrifugation-based ribosome binding assay for IF2 (C), RF3 (F) and LepA (I). Lanes: (1) 70S ribosomes, (2) GTPase, (3) 70S/GTPase, (4) 70S/GTPase/GDPNP, (5) 70SΔL12/GTPase/GDPNP, (6) 70SΔL12/recombinant L12/GTPase/GDPNP, (7) MW standards, (8) 70S control, (9) GTPase control.

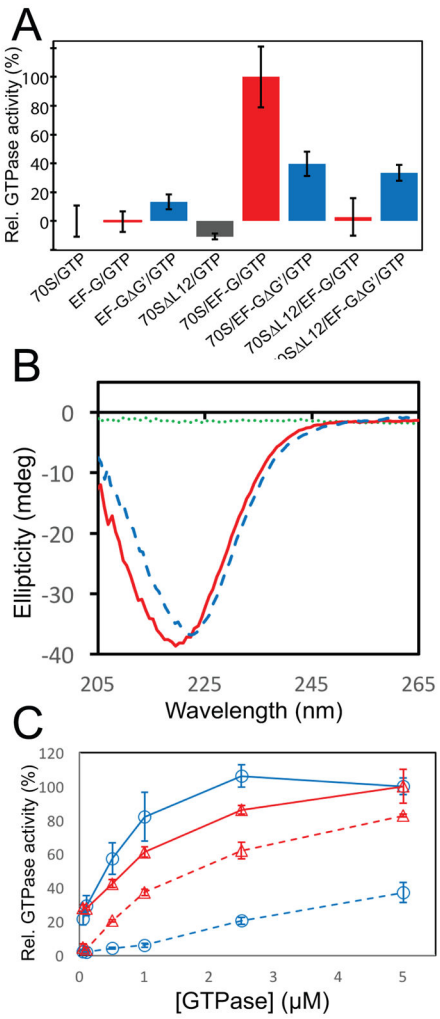

Figure 5. GTP hydrolysis activity for EF-GΔG′ in the presence and absence of ribosomal protein L7/L12

(A) Malachite green GTP hydrolysis activity measured relative to 70S/EF-G/GTP. Red: EF-G, Blue: EF-GΔG′. Single time points were recorded at 60 minutes. Each complex was measured in triplicate and represented as the mean. Error bars represent the standard deviation from the mean. (B) Far UV CD spectra for EF-G (red solid line), EF-GΔG′ (blue dashed line), and buffer (green dotted line). (C) Malachite green GTPase activity for titrations of EF-G and LepA relative to 0.2 μM 70S or 70SΔL12. Open circles (blue): EF-G, open triangles (red): LepA, solid lines: 70S ribosomes, dashed lines: 70SΔL12 ribosomes. Each data point measured in triplicate and error bars represent standard deviations from the mean.